Chapter 5

NOVEL HIV NEUTRALIZING ANTIBODIES SELECTED FROM PHAGE DISPLAY LIBRARIES

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1. INTRODUCTION

Neutralizing antibodies play a major role in host defense against viral infections. Passive administration of antibodies specific for HIV-1 can protect monkeys from infections mediated by the HIV-1 envelope glycoprotein (Env) in a concentration dependent manner (Shibata et al., 1999; Baba et al., 2000; Ruprecht et al., 2001; Xu et al., 2002; Veazey et al., 2003; Burton, 2002; Ferrantelli and Ruprecht, 2002; Mascola et al., 1999; Mascola et al., 2000; Mascola, 2002; Parren et al., 2001). In some of these experiments human monoclonal antibodies (hmAbs) were used that exhibit potent and broad HIV neutralizing activity in vitro (Burton, 1997; Burton, 2002; Ferrantelli and Ruprecht, 2002). Recent clinical trials found that two of these broadly HIV neutralizing hmAbs (nhmAbs), 2F5 and 2G12, could produce a modest decrease in viral load without side effects in humans (Armbruster et al., 2002; Stiegler et al., 2002). However, the potency of 2F5 and 2G12 used in combination in this clinical trial was not sufficient to reduce the HIV-1 plasma RNA levels to the low levels observed after treatment with HAART (Stiegler et al., 2002). Increases in the potency of the currently available broadly HIV nhmAbs and the development of new neutralizing hmAbs might be helpful here although problems associated with

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neutralization escape are likely to be severe in any attempts to use antibodies therapeutically. Importantly, finding immunogens that are able to elicit broadly HIV nhmAbs could be facilitated by the exploration of the interaction of these antibodies with the Env - an approach known as "retrovaccinology" (Burton, 2002). However, only a few broadly cross-reactive HIV nhmAbs have been identified to date and efforts to use mimetics of their epitopes or portions of the epitopes as immunogens are ongoing but of limited success so far (Zwick et al., 2001a). The identification of new broadly cross-reactive HIV nhmAbs and their conserved epitopes is therefore of obvious importance for the development of effective HIV vaccines.

HIV specific polyclonal antibodies can be isolated from the serum of humans infected with HIV or immunized with HIV antigens, and monoclonal antibodies can be produced by hybridomas or identified by screening of phage display libraries (Parren and Burton, 1997). During the last decade the development and screening of libraries from displayed Fabs or scFvs linked to their genotype has become a major methodology for identification of high affinity hmAbs. One of the most potent and well characterized broadly HIV nhmAbs, IgG1 b12, that recognizes the gp120 subunit of the Env was identified by screening of a human antibody phage display library and shown to neutralize a variety of primary HIV-1 isolates (Burton et al., 1994). Recently, another broadly cross-reactive HIV nhmAb, Z13, that recognizes the gp41 subunit of the Env, was also identified by screening of an antibody phage display library (Zwick et al., 2001b). Only three other potent broadly HIV nhmAbs, 2G12, 2F5 and 4E10, have been described until recently. Here, we review our recent work on identification and characterization of new HIV-1 nhmAbs selected from phage display libraries that could contribute to the development of new treatments and vaccines against HIV.

2. PHAGE DISPLAY METHODOLOGY

Antibody fragments, as Fabs or single chain Fv (scFv), have been among the first proteins to be displayed on the surface of a filamentous bacteriophage (McCafferty et al., 1990). In antibody phage display, antibody V-gene repertoires are batch-cloned into the phage genome as a fusion to the gene encoding one of the phage coat proteins (pIII, pVI or pVIII). Upon expression, the coat protein fusion is incorporated into new phage particles that are assembled in the periplasmic space of infected bacteria. Expression of the fusion product and its subsequent incorporation into the mature phage coat results in the surface-displayed recombinant antibodies, while its

genetic material resides within the phage particle. The connection between antibody genotype and phenotype allows the enrichment of phage particles specific for a given antigen using selection based on antigen-antibody interactions. The success of any specific antibody isolation depends largely on the antibody gene repertoire complexity, antibody display methodology, selection procedure (biopanning) and characterization of specific antibody clones (screening).

2.1 Phage display libraries

Antibody libraries can be produced either from antibody V-gene repertoires derived from human or animal donors or from synthetic or semi-synthetic antibody V-gene repertoires constructed in vitro. Libraries made from immunized animals (immune libraries) compared to libraries (naïve libraries) obtained from antigen-naive animals are biased toward antibody genes encoding antibodies recognizing the target antigens (Marks et al., 1991; Williamson et al., 1993b). Thus specific antibodies can be selected using a relatively small, random combinatorial V-gene library derived from an immunized donor (Clackson et al., 1991; Burton et al., 1991). 'Naïve' libraries should have larger diversity ($>10^{10}$ clones) to isolate antibodies with affinity similar to those selected from relatively small libraries made from immunized donors. Antibodies from 'naïve' repertoires can be produced against self-antigens and have relatively fast off-rates (Griffiths et al., 1993) that may require affinity maturation for improvement of binding (Schier et al., 1996c; Schier et al., 1996a; Schier et al., 1996b; Schier and Marks, 1996). Antibodies to several antigens can also be selected from immune libraries, e.g., a single combinatorial library from an HIV-1 infected patient who had been exposed to other viruses was successfully used to generate human Fabs against a plethora of viruses including HIV-1, CMV, HSV-1, HSV-2, RSV, Rubella and varicella zoster (Williamson et al., 1993a). Human monoclonal Fabs to viral antigens have also been selected from combinatorial IgA libraries (Moreno et al., 1995) and IgM libraries (Toran et al., 1999).

The first Fab phage display library used for selection of anti-HIV hmAbs was prepared from the bone marrow of a 31-year-old homosexual HIV-1-infected male who had been asymptomatic for 6 years (Burton et al., 1991). One of the most potent and broadly HIV neutralizing hmAb Fab, b12, was selected from this library and later converted to IgG1 that exhibited even higher neutralizing activity against primary isolates (Burton et al., 1994). Several other antibody libraries have been prepared from HIV-1-infected patients (Parren and Burton, 1997). An important consideration for selection of patients for preparation of antibody libraries is the existence of high titer

of potent and broadly HIV neutralizing antibodies in their serum. Two libraries were prepared from HIV seropositive donors (FDA2 and DS) whose serum was able to neutralize both T-cell line adapted viruses (TCLA) and selection of primary isolates (PI) (Vujcic and Quinnan, Jr., 1995; Parren et al., 1998). The binding of IgG from both patients to Env expressed at the surface of chronically infected cells (Moulard et al., 2000) was strong (Fig 5.1). The binding of the FDA2 patient serum IgG was significantly higher than IgG prepared from a mixture of 25 patient immunoglobulins (HIVIg).

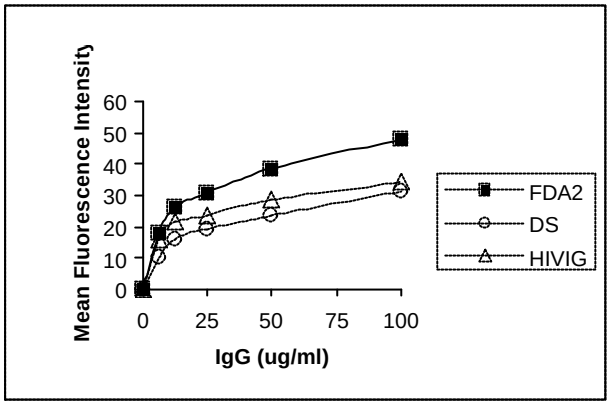

Figure 5.1. Binding capacity of immunoglobulin preparations to infected cells. T-cell line H9 was chronically infected with the TCLA X4 HIV-1_{MN} isolate. Infected cells were immunolabeled with purified IgGs from FDA2, DS or from a pool of sera (HIVIg) at the indicated concentrations. The amount of bound antibodies was measured by flow cytometry and represented in arbitrary units of mean fluorescence intensity.

Purified immunoglobulins from FDA2 sera were also shown to bind to viral particles very efficiently in an assay described recently (Poignard et al., 2003). Purified immunoglobulins from FDA2 and HIVIg were used and compared to anti-HIV-1 hmAbs for binding to the virus (Fig.5.2). Mabs IgG1b12 (CD4-dependent), 2G12 (CD4-independent), and 17b (CD4-induced) were used as control. While the binding of the viral particle to IgG1b12 was inhibited by CD4 preincubation with the virus, the binding to 17b was enhanced by CD4 preincubation in the same experimental condition. As expected binding of the virus to the mAb 2G12 was unaffected. The amount of captured virus was more significantly increased with the FDA2 derived immunoglobulins than with the bulk from HIVIg. The results from the virus capture assay further argue for the presence of antibodies with high affinity for the HIV-1 gp120.

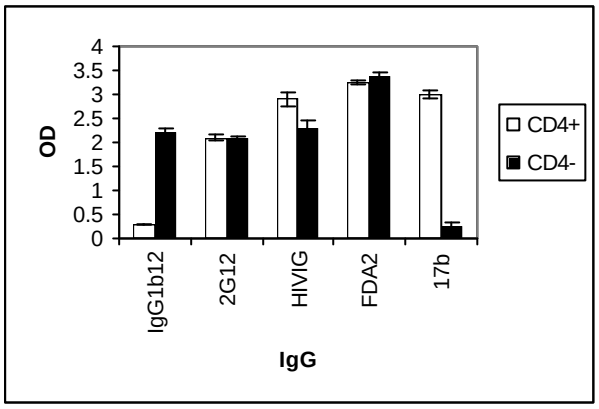

Figure 5.2. Virus capture assay. HIV-1_{MN} was captured on ELISA plates coated with goat anti-human IgG Fc-specific Ab and anti-gp120 Abs or IgG from FDA2 or HIVIg serum. The quantity of p24 captured on the plate was measured as described in (Poignard et al., 2003).

Taken together, the data supported the hypothesis that FDA2 serum contains high concentration of antibodies that are able to bind efficiently Envs. A phage display library from FDA2 was prepared by conventional procedures (Barbas et al., 2001) and used for selection of X5 as described below. Another library was constructed using bone marrow obtained from three long term nonprogressors whose sera exhibited the broadest and most potent HIV-1 neutralization among 37 HIV-infected individuals (T. Evans et al., in preparation) and used for selection of m12,14,16,18 and 20 as summarized below.

2.2 Biopanning

An effective selection procedure is as important as antibody library construction for successful identification of high-affinity antibodies (Griffiths and Duncan, 1998). Selection of specific binders is usually performed by several cycles of incubation with the target antigen and amplification of the recombinant phage, a process referred as “biopanning”. Biopanning allows phage to associate with the target antigen followed by extensive washing to remove non-specifically bound phage, then elution of the remaining particles, and reinfection of *E. coli* to monitor the recovery. Amplification of the eluted phage, followed by repetition of the selection process, allows enrichment of specific-binding clones (Barbas, III et al., 1991). Four to six rounds of panning are usually required to select phage of interest (Barbas et al., 2001).

Many different selection methods have been described, including biopanning on immobilized antigen coated onto solid supports such as ELISA plates, immunotubes or magnetic beads (solid-phase panning) (Clackson et al., 1991; Griffiths et al., 1994; Duenas et al., 1996; Marks et al., 1991; Sawyer et al., 1997), selection in solution using biotinylated antigen (solution-phase panning) (Hawkins et al., 1992), panning on fixed prokaryotic cells (Bradbury et al., 1993) or on mammalian cells (Cai and Garen, 1995) including cultured cells (Li et al., 2001) and primary cells (Ditzel et al., 2000; Williams et al., 2002), subtractive selection using sorting procedures, enrichment on tissue sections or pieces of tissue (de Kruif et al., 1995; Van Ewijk et al., 1997) and, in principle, selections using living animals (Trepel et al., 2002). Solid-phase panning and solution phase panning are commonly used if purified antigen is available. The choice of selection procedure depends on the properties of the targets and antibodies to be isolated. Panning against target antigen coated on magnetic beads is a useful approach for selection of antibodies specific for membrane glycoproteins (Sawyer et al., 1997). Strategies including epitope masking or specific blocking were shown to improve the selection process (Ditzel, 2002; Ditzel et al., 1995; Messmer and Thaler, 2001). An automated screening procedure has also been described recently (Hallborn and Carlsson, 2002).

In most cases one antigen is used in all rounds. Many viruses, including HIV, undergo rapid mutations as one of the strategies to escape host immune surveillance. To select for broadly cross-reactive antibodies recognizing conserved epitopes we hypothesized that selection of high-affinity antibodies against such epitopes can be facilitated by sequentially changing the antigen during the panning of phage display libraries and developed a methodology termed sequential antigen panning (SAP) (Zhang and Dimitrov, 2002, in preparation). This methodology was used for selection of new broadly HIV reactive hmAbs, m6,9,12,14,16,18 and 20, as described below.

3. NEW HIV NEUTRALIZING HUMAN MONOCLONAL ANTIBODIES

3.1 Antibodies Selected by Soluble Recombinant Monomeric gp120ΔV3$_{JRFL}$

Recombinant gp120ΔV3 from HIV-1$_{JRFL}$, a gp120 which has been deleted for the V3-loop, was selected as target for the panning. A number of Fabs have been selected after five rounds of selection/amplification panning

of the FDA2 library on gp120ΔV3$_{JRFL}$ and partially characterized. The CDR3 region of the heavy chains from positive clones

Ia3	AKPTYYDMLSGRSRHYYYMDV
Ia7	AAFRQWFGGLSGVFDS
II105	AKPSYYDMMSGRSRHYSYMDV
II116	DGSKWSRERKLFAPRARNFYYLD
Ia4	GPNERHWGSYRALYFES
Ib9	ASFRQWFGGLSGVFDS
II117	AAFDQWFGGLSGVYDS

Figure 5.3. CDR3 regions of Fabs selected from the panning of the FDA2 library with the gp120ΔV3 JRFL

were sequenced (Fig 5.3). Binding of these Fabs to both monomeric gp120 and trimeric gp120 on chronically infected cells was of high affinity as estimated by ELISA and flow cytometry. Fabs Ia7 and Ia3 neutralized HxB2 TCLA pseudotyped virus (Fig 5.4), but not PIs. Thus the most interesting Fab is probably Ia7 which has been further investigated. Fab Ia7 is directed against the CD4-BS on gp120 as it does compete for the binding of the virus to IgG1 b12 (data not shown).

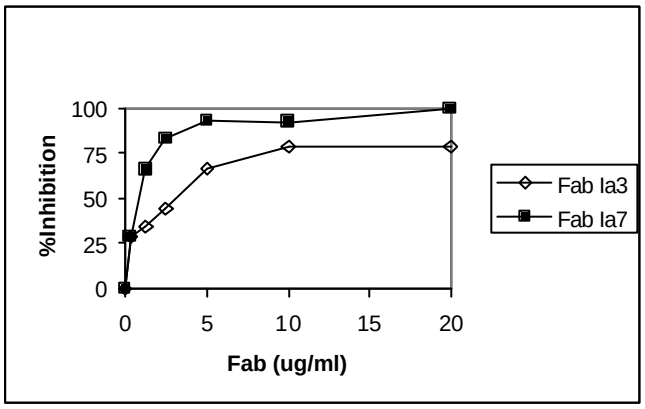

Figure 5.4. Neutralization of the HxB2 TCLA pseudotyped virus. HxB2, a TCLA virus was pseudotyped using the luciferase reporter assay and assayed for neutralization with Ia3 and Ia7 Fabs.

3.2 Antibodies Selected for Binding to $gp120_{JRFL}$-CD4-CCR5 Complexes

HIV enters cells by binding to receptor molecules (CD4 and coreceptors, mainly CCR5 and CXCR4) which induce conformational changes in the Env (Dimitrov, 2000). We hypothesized that these conformational changes could enhance the exposure of conserved epitopes that might be targets for broadly neutralizing antibodies (Dimitrov, 1996; Moulard et al., 2002). The FDA2 library was panned on beads associated with CD4-gp120-CCR5 complexes. A gp120 specific Fab, X5, was selected after the fifth round of panning and was found to bind specifically to gp120 with high affinity (in the nanomolar range (Moulard et al., 2002)). Neutralization experiments demonstrated the potent and broad neutralizing activity of Fab X5 comparable to that of IgG1 b12 for more than 40 primary HIV isolates tested (Fig 5.5 and data not shown).

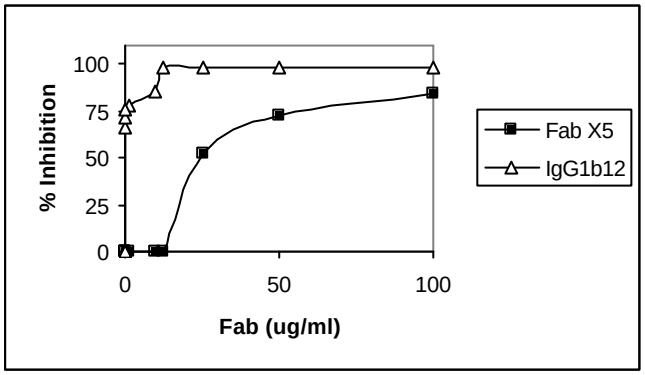

Figure 5.5. Neutralization of HIV-1HxB2 by the Fab X5 and IgG1b12. H9 cells were infected in the presence of IgG1b12 or Fab at indicated concentrations. Virus replication was assessed after 7 days by p24 ELISA measurement.

Recently, X5 was crystallized, its structure determined, and its epitope localized by molecular docking and alanine scanning mutagenesis (Xinhua et al., in preparation). The X5 epitope is located close proximity to the CD4 and coreceptor binding sites. The contact amino acid residues are highly conserved suggesting possible explanation for the breadth of neutralization.

3.3 Antibodies Selected by Sequential Antigen Panning (SAP)

We hypothesized that selection of high-affinity antibodies against conserved epitopes might be facilitated by sequentially changing the antigen during the panning of phage display libraries. We further hypothesized that the use of Env-CD4 complexes in combination with Envs alone as antigens may allow the identification of X5-like antibodies due to the highly conserved nature of the X5 epitope, the X5 high affinity binding to Env alone and its increased binding to Env-CD4 complexes. Five new HIV-specific antibodies (m12,14,16,18,20) were selected from an human Fab phage display library by using SAP against $gp140_{89.6}$–sCD4, $gp140_{IIIB}$-sCD4, $gp140_{89.6}$ and $gp140_{IIIB}$ followed by screening of individual clones with $gp140_{89.6}$, $gp120_{JR-FL}$ and $gp140_{IIIB}$, and their complexes with sCD4 (Zhang et al., in preparation). Some of these antibodies (m14 and m18) bound to Envs from several HIV isolates with high (nM) affinity, and inhibited virus entry and membrane fusion mediated by Envs of selected primary HIV isolates; the neutralization activity of these and other antibodies for a larger panel of isolates is being evaluated. The results suggest that the SAP is effective and could be used for identification of antibodies to conserved epitopes on rapidly mutating viruses or cells.

We used the same technology (SAP) in combination with random mutagenesis to further increase the breadth and potency of X5. Two scFvs (m6,9) were selected that exhibited several fold higher inhibitory activity to more than 30 primary HIV isolates compared to scFv and Fab X5 (Zhang et al., in preparation). These results may have implications for development of novel HIV inhibitors and vaccines.

4. CONCLUSIONS

Phage display methodology has been successfully used for selection of high-affinity, potent, broadly HIV-1 nhmAbs. X5 binds to its epitope with an affinity that is enhanced by the Env interaction with CD4 but is not affected by CCR5; Fab X5 exhibits potent and broad neutralizing activity. The potency and breadth of X5 neutralizing activity was further enhanced by using random mutagenesis in combination with SAP; two scFvs, m6 and m9, selected by this approach exhibited higher neutralization activity than Fab and scFv X5. SAP was also used for selection of five new hmAb Fabs, two of which, m14 and m18, bind to epitopes close to the CD4 binding site, and three Fabs, m12, m16 and m20, bind to epitopes which are exposed better after the gp120 binding to CD4. The neutralizing activity of these antibodies

is currently being evaluated. These results may have implications for development of novel HIV inhibitors and vaccines as well as for elucidation of the mechanisms of HIV entry into cells.

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